

CONTINUATION APPLICATION
FOR
UNITED STATES LETTERS PATENT

TITLE: CANOLA OIL FROM SEEDS WITH REDUCED
GLUCOSINOLATES AND LINOLENIC ACID

APPLICANT: LORIN R. DEBONTE, WILLIE H-T LOH AND ZHEGONG
FAN

CERTIFICATE OF MAILING BY EXPRESS MAIL

Express Mail Label No. EL734685051US

I hereby certify under 37 CFR §1.10 that this correspondence is being deposited with the United States Postal Service as Express Mail Post Office to Addressee with sufficient postage on the date indicated below and is addressed to the Commissioner for Patents, Washington, D.C. 20231.

December 27, 2001
Date of Deposit


Signature

Vince Defante
Typed or Printed Name of Person Signing Certificate

TITLE

A CANOLA VARIETY PRODUCING A SEED
WITH REDUCED GLUCOSINOLATES AND LINOLENIC ACID
YIELDING AN OIL WITH LOW SULFUR, IMPROVED SENSORY
5 CHARACTERISTICS AND INCREASED OXIDATIVE STABILITY

TECHNICAL FIELD

This invention relates to improved canola seeds,
plants and oil having advantageous properties, that is,
a low glucosinolates content and a very low α -linolenic
10 acid ($C_{18:3}$) content, which produce an oil with low
sulfur content, improved sensory characteristics and
oxidative stability.

1. Background

A need exists for an improved vegetable oil with a
15 significantly extended shelf life and greater heat
stability relative to generic canola oil and a positive
nutritional contribution to animal, including human,
diets.

Canola oil has the lowest level of saturated fatty
20 acids of all vegetable oils. "Canola" refers to
rapeseed (Brassica) which has an erucic acid ($C_{22:1}$)
content of at most 2 percent by weight based on the
total fatty acid content of a seed, preferably at most
0.5 percent by weight and most preferably essentially 0
25 percent by weight and which produces, after crushing,
an air-dried meal containing less than 30 micromoles
(μmol) per gram of defatted (oil-free) meal. These
types of rapeseed are distinguished by their edibility
in comparison to more traditional varieties of the
30 species.

As consumers become more aware of the health
impact of lipid nutrition, consumption of canola oil in
the U.S. has increased. However, generic canola oil
cannot be used in deep frying operations, an important
35 segment of the food processing industry.

Canola oil extracted from natural and previously commercially useful varieties of rapeseed contains a relatively high (8%-10%) α -linolenic acid content ($C_{18:3}$) (ALA). This trienoic fatty acid is unstable and easily oxidized during cooking, which in turn creates off-flavors of the oil (Gailliard, 1980, Vol. 4, pp. 85-116 In: Stumpf, P. K., ed., The Biochemistry of Plants, Academic Press, New York). It also develops off odors and rancid flavors during storage (Hawrysh, 1990, Stability of canola oil, Chapter 7, pp. 99-122 In: F. Shahidi, ed. Canola and Rapeseed: Production, Chemistry, Nutrition, and Processing Technology, Van Nostrand Reinhold, New York). One such unsatisfactory species heretofore has been Brassica napus, i.e., spring canola, a type of rapeseed.

It is known that reducing the α -linolenic content level by hydrogenation increases the oxidative stability of the oil. Hydrogenation is routinely used to reduce the polyunsaturates content of vegetable oils, thereby increasing its oxidative stability. The food industry has used hydrogenation to raise the melting point of vegetable oils, producing oil-based products with textures similar to butter, lard and tallow. Trans isomers of unsaturated fatty acids are commonly produced during hydrogenation. However, the nutritional properties of trans fatty acids mimic saturated fatty acids, thereby reducing the overall desirability of hydrogenated oils (Mensink et al., New England J. Medicine N323:439-445, 1990; Scarth, et al., Can. J. Pl. Sci., 68:509-511, 1988). Canola oil produced from seeds having a reduced α -linolenic acid content would be expected to have improved functionality for cooking purposes with improved nutritional value, and therefore have improved value as an industrial frying oil.

However, in general, very little variation exists for α -linolenic acid content in previously known canola quality *B. napus* germplasm (Mahler et al., 1988, Fatty acid composition of Idao Misc. Ser. No. 125). Lines with levels of α -linolenic acid lower than that of generic canola oil are known, but have sensory, genetic stability, agronomic or other nutritional deficiencies. For example, Rakow et al. (J. Am. Oil Chem. Soc., 50:400-403, 1973), and Rakow (Z. Pflanzenzuchtg, 69:62-82, 1973), disclose two α -linolenic acid mutants, M57 and M364, produced by treating rapeseed with X-ray or ethylmethane sulfonate. M57 had reduced α -linolenic acid while M364 had increased α -linolenic acid. However, the instability of the fatty acid traits between generations was unacceptable for commercial purposes.

Brunklaus-Jung et al. (Pl. Breed., 98:9-16, 1987), backcrossed M57 and other rapeseed mutants obtained by mutagenic treatment to commercial varieties. BC₀ and BC₁ of M57 contained 29.4-33.3% of linoleic acid (C_{18:2}) and 4.9-10.8% of α -linolenic acid (C_{18:3}). The oleic acid (C_{18:1}) content was not reported, but by extrapolation could not have exceeded 60%.

Four other lower α -linolenic acid canola lines have been described. Stellar, reported by Scarth et al. (Can. J. Plant Sci., 68:509-511, 1988), is a Canadian cultivar with lower α -linolenic acid (also 3%) derived from M57. Its α -linolenic acid trait was generated by seed mutagenesis. S85-1426, a Stellar derivative with improved agronomic characteristics, also has lower (1.4%) α -linolenic acid (Report of 1990 Canola/Rapeseed Strain Test A, Western Canada Canola Rapeseed Recommending Committee). IXLIN, another lower α -linolenic acid (1.8%) line described by Roy et al. (Plant Breed., 98:89-96, 1987), originated from an

interspecific selection. EP-A 323 753 (Allelix) discloses rape plants, seeds, and oil with reduced α -linolenic acid content linked to limitations in the content of oleic acid, erucic acid, and glucosinolate.

- 5 Another nutritional aspect of rapeseed, from which canola was derived, is its high (30-55 $\mu\text{mol/g}$) level of glucosinolates, a sulfur-based compound. When the foliage or seed is crushed, isothiocyanate esters are produced by the action of myrosinase on glucosinolates.
- 10 These products inhibit synthesis of thyroxine by the thyroid and have other anti-metabolic effects (Paul et al., Theor. Appl. Genet. 72:706-709, 1986). Brassica varieties with reduced glucosinolates content (<30 $\mu\text{mol/g}$ defatted meal) were developed to increase
- 15 the nutritional value of canola meal (Stefansson et al., Can. J. Plant Sci. 55:343-344, 1975). Meal from an ultra-low glucosinolates line, BC86-18, has 2 $\mu\text{mol/g}$ total glucosinolates and significantly improved nutritional quality compared to generic canola meal
- 20 (Classen, Oral presentation, GCIRC Eighth International Rapeseed Congress, Saskatoon, Saskatchewan, July 9-11, 1991). Neither its fatty acid composition nor its seed glucosinolates profile is known.

- 25 There remains a need for an improved canola seed and oil with very low α -linolenic levels in the oil and low glucosinolates in the seed to significantly reduce the need for hydrogenation. The α -linolenic content of such a desirable oil would impart increased oxidative stability, thereby reducing the requirement for
- 30 hydrogenation and the production of trans fatty acids. The reduction of seed glucosinolates would significantly reduce residual sulfur content in the oil. Sulfur poisons the nickel catalyst commonly used for hydrogenation (Koseoglu et al., Chapter 8, pp. 123-
- 35 148, In: F. Shahidi, ed. Canola and Rapeseed:

FOI 10034698-122701

Production, Chemistry, Nutrition, and Processing Technology, Van Nostrand Reinhold, New York, 1990). Additionally, oil from a canola variety with low seed glucosinolates would be less expensive to hydrogenate.

5 2. Summary of the Invention

 This invention comprises a Brassica napus canola yielding seed having a total glucosinolates content of about 18 $\mu\text{mol/g}$ or less of defatted, air-dried meal; the seed yielding extractable oil having 1) an α -
10 linolenic acid content of about 7% or less relative to total fatty acid content of the seed, and 2) a very low sulfur content of less than or equal to 3.00 ppm. The invention also includes a Brassica napus yielding
15 canola oil having, when hydrogenated, a significantly reduced overall room-odor intensity relative to the overall room-odor intensity of generic canola oil. The new variety more particularly yields non-hydrogenated oil significantly reduced in fishy odor relative to the fishy odor of generic canola oil, such odor being
20 characteristic of Brassica seed oil. The seed of such canola variety has an α -linolenic acid content of less than or equal to 7%, more preferably less than or equal to about 4.1% α -linolenic acid ($\text{C}_{18:3}$) relative to total fatty acid content of said seed and a total
25 glucosinolates content of less than 18 $\mu\text{mol/g}$, more preferably less than or equal to about 15 $\mu\text{mol/g}$ and most preferably less than or equal to 13 $\mu\text{mol/g}$ and belongs to a line in which these traits have been stable for both the generation to which the seed
30 belongs and that of its parent.

 This invention further includes processes of making crosses using IMC 01 as at least one parent of the progeny of the above-described seeds and oil derived from said seeds.

10034698-12294

This invention further comprises a seed designated IMC 01 deposited with the American Type Culture Collection, 12301 Parklawn Drive, Rockville, MD, USA 20852 and bearing accession number ATCC 40579, the
5 progeny of such seed and oil of such a seed possessing the quality traits of interest.

3. Detailed Description of the Invention

A spring canola (Brassica napus L.) variety was developed with improved sensory characteristics and
10 oxidative stability in the seed oil. This variety, designated IMC 01, has very low levels of α -linolenic acid ($\text{CH}_3\text{CH}_2\text{CH}=\text{CHCH}_2\text{CH}=\text{CHCH}_2\text{CH}=\text{CH}(\text{CH}_2)_7\text{COOH}$) in the seed oil and very low levels of glucosinolates in the seed. The oil produced from the seed of this variety has very
15 low levels of sulfur and was shown to have significantly improved sensory characteristics over generic canola oils. The IMC 01 is a line in which these traits have been stabilized for both the generation to which the seed belongs and that of its
20 parent generation. Particularly desirable lines of this invention from an agronomic point of view can be derived by conventionally crossing lines producing seeds meeting the definitions of this invention with agronomically well-proven lines such as Westar.

25 In the context of this disclosure, a number of terms are used. As used herein, a "line" is a group of plants that display little or no genetic variation between individuals for at least one trait. Such lines may be created by several generations of self-
30 pollination and selection, or vegetative propagation from a single parent using tissue or cell culture techniques. As used herein, the terms "cultivar" and "variety" are synonymous and refer to a line which is used for commercial production. "Stability" or
35 "stable" means that with respect to the given

component, the component is maintained from generation to generation and, preferably, at least three generations at substantially the same level, e.g., preferably $\pm 15\%$, more preferably $\pm 10\%$, most preferably $\pm 5\%$. The stability may be affected by temperature, location, stress and the time of planting. Comparison of subsequent generations under field conditions should produce the component in a similar manner. "Commercial Utility" is defined as having good plant vigor and high fertility, such that the crop can be produced by farmers using conventional farming equipment, and the oil with the described components can be extracted from the seed using conventional crushing and extraction equipment. To be commercially useful, the yield, as measured by both seed weight, oil content, and total oil produced per acre, is within 15% of the average yield of an otherwise comparable commercial canola variety without the premium value traits grown in the same region. "Agronomically elite" means that a line has desirable agronomic characteristics such as yield, maturity, disease resistance, standability. The amount of fatty acids, such as oleic and linolenic acids, that are characteristic of the oil is expressed as a percentage of the total fatty acid content of the oil. "Saturated fatty acid" refers to the combined content of palmitic acid and stearic acid. "Polyunsaturated fatty acid" refers to the combined content of linoleic and α -linolenic acids. The term "shortening" refers to an oil that is a solid at room temperature. The term "room odor" refers to the characteristic odor of heated oil as determined using the room-odor evaluation method described in Mounts (J. Am. Oil Chem. Soc., 56:659-663, 1979). "Generic canola oil" refers to a composite oil extracted from commercial varieties of rapeseed

10034658 122701

- currently known as of the priority date of this application, which varieties generally exhibited at a minimum 8-10% α -linolenic acid content, a maximum of 2% erucic acid and a maximum of 30 $\mu\text{mol/g}$ total glucosinolate level. The seed from each growing region is graded and blended at the grain elevators to produce an acceptably uniform product. The blended seed is then crushed and refined, the resulting oil being sold for use. Table A shows the distribution of canola varieties seeded as percentage of all canola seeded in Western Canada in 1990.

TABLE A: Distribution of Canola Varieties Grown in Western Canada in 1990

Canola Variety	Percent of Seeded Area
<i>B. campestris</i>	
Candle	0.4
Colt	4.4
Horizon	8.5
Parkland	2.5
Tobin	27.1
<i>B. napus</i>	
Alto	1.1
Delta	0.9
Global	0.9
Legend	18.2
Pivot	0.1
Regent	0.5
Stellar	0.2
Tribute	0.4
Triton	0.7
Triumph	0.2
Westar	29.5
Others	4.4

1004598 12201
T0222T 2594001

Source: Quality of Western Canadian Canola - 1990 Crop Year. Bull. 187, DeClereg et al., Grain Research Laboratory, Canadian Grain Commission, 1404-303 Main Street, Winnipeg, Manitoba, R3C 3G8.

- 5 IMC 01 is a very low α -linolenic acid (<4.1% C_{18:3}) line selected during an extensive germplasm screening effort. Its parentage is unknown. IMC 01 was self-pollinated and selected for low α -linolenic acid (<4.1%) over four consecutive generations. At
10 each generation, seeds from individually pollinated plants were analyzed for fatty acid composition. Data showed no genetic segregation for α -linolenic acid content over five generations of self-pollinations (Table I). Breeder seed was derived from a bulk seed
15 increase of selected plants from the fourth self-crossed generation.

TABLE I: Fatty Acid Composition of IMC 01 Over Five Generations

DATE OF ANALYSIS	PERCENT COMPOSITION				
	C _{16:0}	C _{18:0}	C _{18:1}	C _{18:2}	C _{18:3}
11/87	4.1	1.9	64.1	25.7	1.9
8/88	4.6	2.3	72.6	14.4	2.0
1/89	4.9	1.5	60.4	25.8	2.5
4/89	4.8	1.8	64.3	21.4	4.0
10/89	4.3	2.1	64.1	24.8	2.0

- IMC 01 was planted in replicated field trials in North Dakota, South Dakota, Minnesota, Washington, Idaho and Montana in 1989 and 1990, under both
20 irrigated and nonirrigated conditions. These tests showed that the α -linolenic acid content of IMC 01 was sensitive to temperature (Table II). This was further supported by growing IMC 01 under controlled temperature conditions in growth chambers. Whether or
25 not the observed temperature sensitivity of IMC 01 is

10034698-122701

common to other low α -linolenic acid canola lines is unknown.

A temperature effect on fatty acid compositions has been widely reported in plants, especially oilseed crops (Rennie et al., J. Am. Oil Chem. Soc., 66:1622-1624, 1989). These reports describe general temperature effects on fatty acid composition.

Changes in fatty acid content in seed oil under cool temperatures have been documented in plants such as soybean, peanut and sunflower (Neidleman, In: Proceedings of the World Conference on Biotechnology for the Fats and Oils Industry, Applewhite, T. H., ed., pp. 180-183. Am. Oil. Chem. Soc. 1987).

TABLE II: α -Linolenic acid Content of IMC 01 in Production in 1990

PRODUCTION REGION	\bar{X} TEMPERATURE DURING SEED MATURATION	α -LINOLENIC ACID CONTENT
Eastern Washington	74°F	1.9%
Eastern Washington	74°F	2.0%
Northern Idaho	70°F	3.0%
Northern Idaho	67°F	2.9%
Eastern Idaho	62°F	3.5%
Southern Montana	66°F	4.1%
Central Montana	67°F	4.0%

In addition to very low α -linolenic acid, IMC 01 is also characterized by very low levels of glucosinolates. Glucosinolates are sulfur-based compounds common to all Brassica seeds. Glucosinolates exist in aliphatic or indolyl forms. Aliphatic glucosinolates can be analyzed via gas chromatography (GC) (Daun, Glucosinolate analysis of rapeseed (canola), Method of the Canadian Grain Commission, Grain Research Laboratory, Canadian Grain Commission, Winnipeg, 1981). Indolyl glucosinolates have only

recently been analyzed via high performance liquid chromatograph (HPLC). Prior to the adoption of the HPLC method, total glucosinolates were calculated by multiplying the aliphatic glucosinolates by a correction factor. Canola quality in the seed is defined as having <30 $\mu\text{mol/g}$ of glucosinolates in the defatted meal.

IMC 01 and Westar were tested in five locations in southeastern Idaho in 1990. Three of the locations were irrigated (I) and two were dryland (D) conditions. Table IIIa shows the difference in total aliphatic glucosinolate between IMC 01 and Westar grown at these locations. The aliphatic glucosinolate values are reported as $\mu\text{mol/gm}$ of defatted meal.

The aliphatic glucosinolate content of IMC 01 by location was consistently lower and more stable than that of Westar at all locations tested. The average glucosinolate contents of IMC 01 was 4.9 $\mu\text{mol/gm}$ while Westar was 13.3 $\mu\text{mol/gm}$. A Least Significant Difference (LSD) test was used to determine if the two were significantly different at all locations. IMC 01 was found to be significantly different from Westar at a level of $P < 0.05$.

HPLC analysis of IMC 01 vs Westar, the most widely grown spring canola variety in North America, shows that IMC 01 has much lower levels of aliphatic glucosinolates (Table III). No significant differences exist for indolyl glucosinolates. Glucosinolates content is also subject to environment influence, e.g., sulfur fertility and drought stress. However, IMC 01 consistently had the lowest and the most stable aliphatic glucosinolates levels at all locations tested (Table IV). The locations tested differ in altitude, temperature, fertility, irrigation, and other cultural practices. Among the low α -linolenic canola lines for

1003469-12204

which glucosinates analysis have been performed, IMC 01 has the lowest level of total seed glucosinolates (Table V).

TABLE III: Glucosinolates Profiles of IMC 01 and Westar Varieties

GLUCOSINOLATES ($\mu\text{mol/g}$)	IMC 01	WESTAR
3-butenyl	1.2	4.2
4-pentenyl	0.1	0.2
2-OH-3-butenyl	3.1	7.0
2-OH-4-pentenyl	0.9	0.4
Total Aliphatics	5.3	11.8
4-OH-3-indolylmethyl	6.2	6.1
3-indolylmethyl	0.8	1.0
4-methoxyindolyl	0.1	tr
1-methoxyindolyl	0.1	0.2
Total Indolyls	7.2	7.3
Total Glucosinolates	12.5	19.1

TABLE IV: Aliphatic Glucosinolates of IMC 01 and Westar over Different Environments in Southeastern Idaho

LOCATION*	ALIPHATIC GLUCOSINOLATES CONTENT ($\mu\text{mol/g}$)	
	IMC 01	WESTAR
Newdale - I	4.7	13.0
Soda Springs - D	6.3	9.9
Tetonia - D	5.0	13.5
Tetonia -I	3.7	13.3
Shelley - I	5.0	17.0
Average	4.9	13.3
Standard Deviation	0.93	2.51

*I = Irrigated, D = Dryland

10034698-122701
FOI 2221 86948001

TABLE V: Glucosinolates Content of
Low α -Linolenic acid Canola Varieties and Westar

GLUCOSINOLATES ($\mu\text{mol/g}$)	ALIPHATIC	INDOLYL	TOTAL
IMC 01	5.3	7.2	12.5
Stellar	5.2	19.5	24.7
S85-1426	7.9	13.4	21.3
Westar	11.8	7.3	19.1

- IMC 01 was produced, using normal production practices for spring canola, in Idaho and North Dakota in 1988, in Idaho, Washington State and Montana in 1989, in Idaho, Washington State, Montana, Oregon, and Wyoming in 1990. When grown in suitable environments, where the average daily temperature (high temperature plus low temperature divided by 2) exceeds 20°C, the oil contains <4.1% α -linolenic acid. As an example, a normal fatty acid profile was produced at Casselton, North Dakota. The crop produced in Ashton, Idaho, was subject to extremely cool conditions and had higher levels of α -linolenic acid. The crops obtained from the field tests were crushed and processed to produce refined, bleached and deodorized (RBD) canola oil at the Protein, Oil, Starch (POS) Pilot Plant in Saskatoon, Saskatchewan. A method of bleaching canola oil is provided in the AOCS' Recommended Practice Cc 8a-52 (AOCS Methods and Standard Practices, 4th Edition (1989)). A method for the refining of crude oils is provided in the AOCS Practice Cc 9a-52 (AOCS Methods and Standard Practices, 4th Edition (1989)). The oils were tested at the Vegetable Oil Research Laboratory, U.S.D.A./Northern Regional Research Center, for organoleptic and sensory characteristics.
- Testing to assure that desirable sensory characteristics are obtained in the Brassica napus variety was essential. The evaluation of odors has

been conducted in a variety of ways on low α -linolenic acid canola oils. The testing methods are based on the fact that vegetable oils emit characteristic odors upon heating. For example, Prevot et al. (J. Amer. Oil Chemists Soc. 67:161-164, 1990) evaluated the odors of a French rapeseed, "Westar", and "low linolenic" canola oils in a test which attempted to reproduce domestic frying conditions. In these evaluations the test oils were used to fry potatoes and the odors were evaluated by a test panel. The odor tests showed that the "low linolenic" (approximately 3%) line had a significantly higher (more acceptable) odor score than the French rapeseed and "Westar" lines, which were very similar to each other. Eskin et al. (J. Amer. Oil Chemists Soc. 66:1081-1084, 1989) evaluated the odor from canola oil with a low linolenic acid content, a laboratory deodorized sample, and a commercially deodorized sample by sniffing in the presence of the oil itself. These studies demonstrated that a reduction in the linolenic acid content of canola oil from 8-9% to 1.6% reduced the development of heated odor at frying temperatures. However, the odor of the low linolenic acid oil was still unacceptable when heated in air to a majority of the panelists, suggesting that low linolenic acid alone is not sufficient to guarantee acceptable odor.

Mounts (J. Am. Oil Chem. Soc., 56:659-663, 1979) describe a distinct room-odor evaluation method that is used to reproducibly assess the odor characteristics of a cooking oil upon heating. This is the evaluation method of choice owing to its reproducibility and its approximation of odors emitted upon heating the oil. In this method, the oil is heated in a separate chamber and the odor pumped into the room containing the trained evaluators. As noted elsewhere, where the term "room-odor" is used herein, it refers to this method of

Mounts. This method is distinct from earlier described tests where the oil and evaluator are within the same room. Such same room testing is referred to as "uncontrolled bench top odor tests" and is considered less accurate and less reliable than the Mounts' room odor evaluation method.

The room-odor characteristics of cooking oils can be reproducibly characterized by trained test panels in room-odor tests (Mounts, J. Am. Oil Chem. Soc. 56:659-663, 1979). A standardized technique for the sensory evaluation of edible vegetable oils is presented in AOCS' Recommended Practice Cg 2-83 for the Flavor Evaluation of Vegetable Oils (Methods and Standard Practices of the AOCS, 4th Edition (1989)). The technique encompasses standard sample preparation and presentation, as well as reference standards and method for scoring oils. When heated, generic canola oil has reduced stability and produces offensive room odors. Refined-Bleached-Deodorized (RBD) canola oil is characterized by a fishy flavor in such tests. This characteristic is commonly ascribed to its high polyunsaturated fatty acid content, particularly α -linolenic acid, relative to other vegetable oils. The individual fragrance notes (odor attributes) of the oils are evaluated by Least Significant Difference Analysis. Notes which differ by greater than 1.0 can be reproducibly measured by a sensory panel. In these tests, IMC 01 oil expressed significantly reduced levels of the offensive odors (Table VI).

TABLE VI: Room Odor Intensity of IMC 01
and Generic Canola Oil

ODOR ATTRIBUTES	IMC 01	GENERIC CANOLA OIL
Overall	4.6 ^a	7.4 ^b
Fried Foods	1.8	3.5
Doughy	1.0	0
Fishy	0 ^a	5.5 ^b
Burnt	0 ^a	0.9 ^b
Acrid	0 ^a	2.3 ^b
Woody/Cardboard	1.9	0
Hydrogenated	0	0
Pastry/Sugary	0	0
Waxy	0	0
Chemical	0	0

0 = None; 10 = Strong. Scores with different superscript letters are significantly different ($P < 0.05$). Least Significant Difference for individual odor notes is 1.0. Differences greater than 1.0 can be reproducibly measured by room-odor analysis.

Due to its relatively low stability, canola oil is often hydrogenated for frying. However, hydrogenation produces a characteristic (hydrogenated) room odor which is unacceptable to food manufacturers.

- 5 Surprisingly, hydrogenated IMC 01 oil also has reduced levels of the characteristic hydrogenated room odor (Table VII). Table VII shows that the overall room-odor intensity of hydrogenated IMC 01 is significantly less than that of hydrogenated generic oil, as indicated
- 10 by a difference in scores of greater than 1.0 in standardized flavor evaluation trials.

TABLE VII: Room Odor Intensity and Individual Odor Descriptions for Hydrogenated Canola Oils

ODOR ATTRIBUTE	HYDROGENATED, GENERIC CANOLA SHORTENING	HYDROGENATED IMC 01 SHORTENING (2% α -LINOLENIC ACID)	HYDROGENATED IMC 01 SHORTENING (6.8% α -LINOLENIC ACID)
Overall Intensity	6.6 ^b	3.8 ^a	3.9 ^a
Fried Food	2.7	1.1	1.5
Doughy	1.2	0.6	0.8
Fishy	0.6	0	0
Burnt	0.5	0	0
Acrid	0.8	0	0
Hydrogenated	3.2	1.8	2.3
Waxy	0.5	0.6	0.
Other	4.5 rubbery flowery weedy soapy	2.4 fruity smoky	2.8 fruity flowery sweet pastry

0 = None; 10 = Strong. Scores with different superscript letters are significantly different ($P < 0.05$). Least Significant Difference for individual odor notes is 1.0. Differences greater than 1.0 can be reproducibly measured by room-odor analysis.

IMC 01 produces an oil which has improved sensory characteristics. Such improvements have been predicted for low α -linolenic acid canola oils (Ulrich et al., J. Am. Oil Chem. Soc., 8:1313-1317, 1988). However, the improved sensory characteristics of IMC 01 appears not to be related solely to its low α -linolenic acid content. Surprisingly, IMC 01 canola oils with both high and low levels of α -linolenic acid showed similar degrees of improvement. Sensory tests have shown that IMC 01 oil maintains its improved quality at both 2% and 6.8% α -linolenic acid.

1003458-42204
FOIA b7 c

The very low glucosinolates characteristic of IMC 01 seed is believed to contribute to the improved sensory characteristic of IMC 01 oil. Glucosinolates in the seed are converted to sulfur compounds. Most of the sulfur breakdown products remain in the meal, but some inevitably contaminate the oil. Lower levels of glucosinolates in the seed are believed to result in lower sulfur content in the oil, and this is believed to reduce the objectionable odor characteristics of canola oil (Abraham et al., J. Am. Oil Chem. Soc., 65:392-395, 1988). An analysis of the sulfur content of IMC 01 oil and several generic canola oils has been performed. IMC 01 oil has approximately one-third the sulfur content of leading generic canola oils (Table VIII).

TABLE VIII: Sulfur Content of Canola Oils

Canola Oils	Sulfur Content
Acme Brand Canola Oil	3.8 ppm
Hollywood Brand Canola Oil	3.8 ppm
Puritan Brand Canola Oil	3.9 ppm
IMC 01 Canola Oil	1.3 ppm

The biochemical, molecular and genetic mechanisms responsible for the room-odor quality of vegetable oils are not fully understood. Improvements in vegetable oil processing technology, i.e., preferential removal of sulfur during processing, less abusive oil extraction procedures, minimal processing, gentler deodorization, etc., may improve the overall quality of vegetable oils, including both sensory and functional characteristics (Daun et al., J. Am. Oil Chem. Soc., 53:169-171, 1976). IMC 01 will benefit from any such processing improvements, and will maintain its improved sensory characteristics over generic canola oil under equivalent processing conditions.

IMC 01 is true breeding as are its progeny. The traits responsible for reduced α -linolenic acid and reduced total glucosinolates in the seed which yield an oil low in sulfur having improved sensory

5 characteristics have a genetic basis. The data presented herein show that these traits are stable under different field conditions. These traits can be removed from the IMC 01 background and are transferred into other backgrounds by traditional crossing and
10 selection techniques.

Crosses have been made with IMC 01 as one parent to demonstrate that the superior IMC 01 quality/sensory traits are transferred along with the superior agronomic traits of another parent such as the
15 Canadian canola line, Westar, into descendents. The parent to which IMC 01 is crossed is chosen on the basis of desirable characteristics such as yield, maturity, disease resistance, and standability. Conventional breeding techniques employed in such
20 crossings are well known by those skilled in the art. Thus, a method of using the IMC 01 Brassica napus is to cross it with agronomically elite lines to produce plants yielding seeds having the characteristics listed above.

25 The general protocol is:

- a. cross IMC 01 to a selected parent;
- b. produce a "gametic array" using microspheres of the F_1 plants to produce dihaploid (DH) individuals;
- 30 c. field trial DH_2 individuals for yield and select from IMC 01 α -linolenic acid and glucosinolate levels; and
- d. test selected individuals for oil quality using RBD oil.

Example 3 is a specific example of such work to develop descendents to IMC 01 which retain the desirable quality traits. The data of Example 3 show that the quality traits of IMC 01 are heritable in such crosses.

The present invention is further defined in the following Examples, in which all parts and percentages are by weight and degrees are Celsius, unless otherwise stated. It should be understood that this Example, while indicating preferred embodiments of the invention, is given by way of illustration only. From the above discussion and this Example, one skilled in the art can ascertain the essential characteristics of this invention, and without departing from the spirit and scope thereof, can make various changes and modifications of the invention to adapt it to various usages and conditions.

EXAMPLE 1

IMC 01, originally designated DNAP #336, was grown in a greenhouse in Cinnaminson, New Jersey, over several seasons to select for a stable, very low α -linolenic line. Day/night temperatures from August through December in the greenhouse averaged 80°F/65°F with fluctuations of $\pm 5^\circ\text{F}$, 75°F/65°F from January through April, and 85°F/65°F from March through July. The plants were grown in 1-gallon pots under natural day length, except from October through May when the plants received 14 hours of supplemental lighting. Flowering racemes were covered with paper bags to prevent cross-pollination, and gently shaken to induce seed set. Watering was decreased as pods reached maturity.

For field testing, IMC 01 was planted in multi-location trials and production plots in Montana, Idaho and Washington. The trials were planted in a

completely randomized block design with four replications. Each block contained eight plots of 6 meters by 8 rows. IMC 01 was also planted in large acreages (>25 acres) according to standard agronomic procedures for spring canola production, with a minimum 1/2-mile isolation from other Brassica napus crops. Depending on location, the fields were planted in April or May, and harvested in August or September. Plantings were made on dryland, following both fallow or recrop, or under irrigation. Mature pod samples were taken following swathing for chemical analysis.

For fatty acid analysis, 10-50 seed samples were ground in 15-mL polypropylene tubes and extracted in 1.2 mL 0.25 N KOH in 1:1 ether/methanol. The sample was vortexed for 10 sec and heated for 60 sec and in 60°C water bath. Four mL of saturated NaCl and 2.4 mL of iso-octane were added, and the mixture was vortexed again. After phase separation, 600 µL of the upper organic phase was pipetted into individual vials and stored under nitrogen. One µL sample was injected into a Supelco SP-2330 fused silica capillary column (0.25 mm ID, 30 m length, 0.20 µm df, Bellfonte, PA).

The gas chromatograph was set at 180°C for 5.5 min, then programmed for a 2°C/min increase to 212°C, and held at this temperature for 1.5 min. Chromatography settings were: Column head pressure - 15 psi, Column flow (He) - 0.7 mL/min, Auxiliary and Column flow - 33 mL/min, Hydrogen flow = 33 mL/min, Air flow - 400 mL/min, Injector temperature - 250°C, Detector temperature - 300°C, Split vent - 1/15.

A standard industry procedure for HPLC analysis of glucosinolates was used to analyze the glucosinolates composition of the seed (Daun et al., In: Glucosinolate Analysis of Rapeseed (Canola). Method of

10034698 122701

the Canadian Grain Commission, Grain Research Laboratory, 1981).

IMC 01 seed was harvested and processed to produce refined, bleached and deodorized (RBD) oil. Some oil
5 was hydrogenated after refining, bleaching and deodorization, then redeodorized.

Before extraction, the seed was tempered to adjust the moisture content to 9% and flaked to 0.38 to 0.64 cm in a ribbon blender. The flakes were cooked in a
10 stack cooker at 82.8°C for 30 min (8.5% moisture) and pre-pressed with vertical and horizontal bar spacings set to 0.031 cm, vertical shaft speed at 40 rpm and horizontal shaft at 25 rpm. The press cake was
15 extracted in a Crown Model 2 extractor at 37.3 kg and hexane extracted with a 2:1 solvent to solids ratio.

The crude oil was alkali refined at 65°C-70°C for 30 min with 0.2% to 85% phosphoric acid, then mixed with sodium hydroxide to neutralize free fatty acids. Soaps were removed with a water wash (65°C water, 5
20 min) and the oils bleached with .75% each by weight of Clarion and Acticil bleaching earths for 30 min to remove color bodies. The resulting oil contained no peroxides, 0.08% free fatty acids, and had a Gardner color of 10-.

25 The oil was continuously deodorized at 265°C at 300 kg/h. The steam rate was 1% of feed rate. The deodorized oil was preheated to 68-72°C prior to deaeration. RBD oil was stored in food grade plastic drums or pails at 4°C under nitrogen prior to testing.

30 For hydrogenation, RBD oil was heated to 350°F under vacuum in a stainless steel pressure reactor. A 0.5% sulfur-poisoned nickel catalyst, Englehardt SP-7, was added to the oil at 80.1°C, and hydrogen gas was introduced at 40 psi. Periodic samples were analyzed
35 until an oil with a 30.5°C melting point was achieved.

The hydrogenated oil was redeodorized and stored by the methods described previously.

The RBD and hydrogenated oil samples were analyzed for room-odor characteristics by a trained test panel in comparison with a generic, commercially available RBD canola oil (Proctor & Gamble) and generic, commercially available hydrogenated canola shortening as described previously. The testing protocol used is described in Mounts (J. Am. Oil Chem. Soc. 56:659-663, 1979) which is hereby incorporated by reference. The testing controlled for temperature of the oil, distance from the oil and room volume, and required that the oil was heated in a separate chamber and pumped into the room containing the trained panelist.

Specifically, room odor profiles of IMC 01 and a generic canola oil were obtained as follows:

A. Room Odor Protocol

A 150 mL sample of the selected oil was heated to 190°C for 30 min before the start of each panel test. The oil was maintained at this temperature throughout each session. For each session a fresh oil sample was used.

Panelists visited each odor room for approximately 15 sec. A five min rest was required between visits. Visitation to each odor room was randomized among the panelists.

The trained panelists judged the room odor for intensity of odor, quality of odor, and odor attributes. The intensity was ranked as: 0-4 weak, 5-7 moderate, and 8-10 strong. The quality of odor was judged as: 0-1 bad, 2-3 poor, 4-6 fair, 7-8 good, and 9-10 excellent. The odor attributes were ranked as: 0-1 bland, 2-4 weak, 5-7 moderate, and 8-10 strong. The flavor attributes were fried, painty, fishy,

hydrogenated, burnt, cardboard, metallic, rubbery, waxy, buttery, and nutty.

B. Generic Oil - IMC 01 Profile Comparison

- 5 A generic, commercially available canola oil (Proctor & Gamble) was used in the IMC room odor tests as the standard or generic canola oil. In a comparative test, the standard canola oil was significantly ($P < 0.05$) higher in room odor intensity
- 10 than IMC 01 (Table IX). The standard canola oil odor was of "moderate" intensity while the IMC 01 was considered "weak". The overall quality of the IMC 01 room odor was significantly ($P < 0.05$) better than the standard canola oil. The standard canola oil had
- 15 significantly ($P < 0.05$) higher intensities for fried, painty, and cardboard odors than the IMC 01 oil.

100493 100493 100493

Table IX: Room Odor Profile of Generic
(Proctor & Gamble) and IMC 01 Oil

Evaluation*	IMC 01	Generic
A. Intensity	3.4 ^a	5.2 ^b
B. Quality	5.8 ^a	4.9 ^b
C. Odor Attributes		
Fried	1.9 ^a	2.9 ^b
Painty	0.4	1.3
Fishy	0.8 ^a	1.9 ^b
Hydrogenated	1.1	0.6
Rancid	0.7	0.9
Burnt	0.8	1.4
Cardboard	0.1 ^a	1.5 ^b
Metallic	0.5	0.1
Rubbery	0.0	0.0
Waxy	0.6	0.0
Buttery	0.7	0.3

* The "intensity" was ranked as: 0-4 weak, 5-7 moderate, and 8-10 strong. The "quality" of odor was judged as: 0-1 bad, 2-3 poor, 4-6 fair, 7-8 good, and 9-10 excellent. The "odor attributes" were ranked as: 0-1 bland, 2-4 weak, 5-7 moderate, and 8-10 strong.

Scores with different superscript letters are significantly different ($P < 0.05$).

- Pilot plant-processed samples of Example 2 generic canola (low erucic acid rapeseed) oil and oil from IMC 01 canola with the fatty acid compositions modified by mutation breeding and/or hydrogenation were evaluated for frying stability α -linolenic acid contents were 10.1% for generic canola oil, 1.7% for canola modified by breeding (IMC 01) and 0.8% and 0.7% for IMC 01 oils modified by breeding and hydrogenation. The IMC 01 modified oils had significantly ($P < 0.05$) less room odor intensity than the generic canola oil

after initial heating tests at 190°C as judged by a sensory panel under conditions of AOCS Cg 2-83. The generic canola oil had significantly higher intensities for fishy, burnt, rubbery, smoky, and acrid odors than the modified oils. Foam heights of the modified oils were significantly ($P < 0.05$) less than those of the generic oil after 20, 30 and 40 hrs of heating and frying at 190°C. The flavor quality of french fried potatoes was significantly ($P < 0.05$) better for all the potatoes fried in modified oils than those fried in generic canola oil. The potatoes fried in generic canola oil were described by the sensory panel as fishy. No off-flavors were detected in potatoes fried in the modified oils.

15

EXAMPLE 3

This Example demonstrates that the traits of very low α -linolenic acid and very low glucosinolate content are transferred to IMC 01 progeny.

FOOTNOTES

↓
F₁ ← Gametic Array
↓

↓ ← Select Line HW3.001

↓	↓
Greenhouse (DH ₃)	Isolation Tents (DH ₃)
Single Plants	California
ALA Range: 2.5-2.8%	ALA Content: 2.5%
	Glucosinolates: 10 <μmole/gm

Year 4

↓

Pre-Production Increase (DH₄)

Idaho

ALA 2.1%

The pre-production will be crushed and the oil refined for quality.

Once a canola line has been stabilized, fully conventional methods of plant biotechnology, breeding and selection are used to further enhance, for example, the agronomic properties of the resultant line in order to improve important factors such as yield, hardiness, etc. Such techniques are also well known and include, e.g., somaclonal variation, seed mutagenesis, anther and microspore culture, protoplast fusion, etc. See, e.g., Brunklaus-Jung et al., Pl. Breed., 98:9-16, 1987; Hoffmann et al., Theor. Appl. Genet., 61, 225-232 (1982), each herein incorporated by reference).

A deposit of seed designated IMC 01 has been made in the American Type Culture Collection (ATCC) depository (Rockville, MD 20852) and bears accession number ATCC 40579. The deposit was made on 2 March 5 1989 under conditions complying with the requirements of the Budapest Treaty.

EXAMPLE 4

IMC 01 was compared to Alto, a "generic" variety of commercial canola oil.

Oils were processed using standard commercial refining, bleaching and deodorizing processes.

TABLE X

Processed Oil Analysis

	<u>IMC 01</u>	<u>Alto</u>
Red color	0.5	0.7
Yellow color	4	5
para-anisidine value	0.97	2.32
Peroxide value, meg/g	0.6	0.7
TOTOX value - p-av + 2(pv)	2.07	3.72
Total Polymers, %	0.02	0.01
Total Polar Material, %	0.77	0.36
Free fatty acid, %	0.022	0.013
Fatty acid composition, %		
C16:0	4.2	4.0
C18:0	2.2	2.0
C18:1	63.5	62.5
C18:2	22.2	18.3
C18:3	4.9	7.7

- 10 Results: Chemical analysis indicates oils were processed within industry standards for quality vegetable oils (see Table X). Based on AOCS Recommended Practice Cg 3-91; oils with a TOTOX value of less than 4.0 indicate good stability. TOTOX value
- 15 = para-anisidine value + 2(peroxide value).

T0422T 8694E00T

Colors determined by American Oil Chemists' Society (AOCS) method Cc 13b-43, using American Oil Tintometer, Model AF715, The Tintometer LTD., Salisbury, England.

- 5 para-Anisidine Value (p-av) determined by AOCS method Cd 18-90, measures secondary oxidation by-products in oils.

Peroxide Value determined by AOCS method Cd 8b-90, measures the primary oxidation product in oils.

- 10 Total polymers determined by AOCS method Cd 22-91, gel-permeation HPLC.

Total Polar Materials determined by AOCS method Cd 20-91, packed column method adapted to HPLC.

- 15 40. Free fatty acid determined by AOCS method Ca 5a-

Fatty acid composition determined by AOCS method Ce 1e-91, capillary gas liquid chromatography.

Oxidative Stability by Oxidative Stability Instrument (OSI)

- 20 Oxidative stability measured by Automatic Oxygen Method (AOM) hours determined by American Oil Chemists Society (AOCS) method Cd 12b-92, Fat Stability, Oil Stability Index (OSI), using an Oxidative Stability Instrument, manufactured by Omnion/Archer Daniels
25 Midland, Decatur, Illinois.

	<u>IMC 01</u>	<u>Alto</u>
AOM hours	26.8	17.5

Results indicate IMC 01 has greater AOM hours and therefore greater oxidative stability than Alto.

Oxidative Stability and Flavor Stability by Accelerated Aging

- 30 The Schaal oven method of accelerated aging is used in the oil industry to measure the oxidative and flavor stability of oil. The Schaal oven method

10034698-12204
FOI 227 854E007

involves examining samples of oil at predetermined intervals held at 60°C in the dark. One day under Schaal oven conditions is equivalent to one month storage in the dark at ambient temperature.

- 5 Oxidative stability is measured by monitoring the increases in peroxides (peroxide value) and secondary oxidative by-products (para-anisidine value) in the oils held at 60°C for twelve days.

- 10 Flavor stability is determined by a trained sensory panel using the same oils tested for oxidative stability.

Sample preparation:

- 15 400 g of oil placed in 500 mL amber glass bottles (80 mm wide, 140 mm high, with a 42 mm opening), uncapped, held in 60°C (range 59 to 61°C) convection oven (Blue M, manufactured by Blue M Electric) for 3, 6, 9 and 12 days. One bottle of oil per day per type of oil.

- 20 Samples were frozen immediately after removing from the oven. Peroxide value, para-anisidine value and sensory evaluation were made within seven days after samples were frozen.

Oxidative Stability by Accelerated Aging

TABLE XI
Changes in Peroxide Value and para-Anisidine Value

<u>Days</u>	<u>PV</u>		<u>p-AV</u>	
	<u>IMC 01</u>	<u>Alto</u>	<u>IMC 01</u>	<u>Alto</u>
0	.6	0.7	0.97	2.32
3	0.9	5.33	0.99	2.52
6	5.48	11.8	1.36	5.02
9	10.3	15.7	2.45	6.5
12	14.1	18.8	3.42	7.49

(see Figures 1 and 2)

10034698-122701

Results: Based on increases in peroxide and p-av values over the 12 day storage period, IMC 01 has greater oxidative stability than Alto (see Table XI).
Flavor Stability by Accelerated Aging:

- 5 Sensory panel trained in evaluation of vegetable oils according to protocol of AOCS method Cg 2-83, Flavor Panel Evaluation of Vegetable Oils. Using AOCS flavor standards panel members were trained to identify the following off flavors; fishy, green, cardboard,
 10 plastic and painty.

TABLE XII
Overall Acceptance Scores
and Total Off-Flavor Intensities

Day	<u>Overall Acceptance¹</u>		<u>Total Off-Flavors²</u>	
	<u>IMC 01</u>	<u>Alto</u>	<u>IMC 01</u>	<u>Alto</u>
0	7.53	6.01	0.59	2.32
3	7.04	3.2	0.87	8.29
6	5.44	3.10	4.17	9.46
9	3.81	1.78	6.22	10.15
12	4.00	2.00	7.16	12.89

¹Overall acceptance scores are based on a 0-9 scale; 0 is extremely unacceptable and 9 is very acceptable.

²Total off-flavor intensities include fishy, green, cardboard, and painty. Ratings are based on a 0-15 scale, where 0 is bland and 15 is very intense.

Results:

Overall acceptability scores were significantly different after 0, 3, 6, 9, and 12 days ($p=0.05$) (see Table XII).

- 15 Correlation between overall acceptance score and total off-flavors $r^2 -0.9485$ (see Figure 5).

Significantly lower overall acceptance scores and lower total off-flavor intensities indicates IMC 01 has significantly better flavor stability than Alto.

EXAMPLE 5

IMC 02 was compared to Stellar, a commercially available low alpha linolenic variety of canola.

TABLE XIII
Processed Oil Analysis

	<u>IMC 02</u>	<u>Stellar</u>
Red color	0.2	0.3
Yellow color	3	3
para-Anisidine value	0.48	0.78
Peroxide value, meq/g	0.2	0.6
TOTOX value = p-av + 2 (pv)	0.88	1.98
Total Polymers, %	0.01	0
Total Polar Material, %	0.63	0.42
Free fatty acid, %	0.026	0.014
Fatty acid composition, %		
C16:0	3.8	4
C18:0	2.2	2.3
C18:1	66.3	62.8
C18:2	22.3	24.4
C18:3	2.7	3.3

- 5 Results: Chemical analysis indicates oils were processed within industry standards for quality vegetable oils (see Table XIII). Based on AOCS Recommended Practice Cg 3-91; oils with a TOTOX value of less than 4.0 indicate good stability. TOTOX value = para-anisidine value + 2(peroxide value).

Oxidative Stability - OSI AOM Hours

	<u>IMC 02</u>	<u>Stellar</u>
AOM hours	31.5	23.1

- 10 Results indicate IMC 02 has greater AOM hours and therefore greater oxidative stability than Stellar.

Oxidative Stability by Accelerated Aging

TABLE XIV
Changes in Peroxide Value and para-Anisidine Value

<u>Days</u>	<u>PV</u>		<u>p-AV</u>	
	<u>IMC 02</u>	<u>Stellar</u>	<u>IMC 02</u>	<u>Stellar</u>
0	0.2	0.6	0.48	0.78
3	0.7	1.39	0.76	0.84
6	1.5	3.77	0.26	0.99
9	4.4	7.37	0.82	2.07
12	9.6	13.3	1.18	2.56

(See Figures 6 and 7)

Results: Based on increases in peroxide and p-av values over the 12 day storage period, IMC 02 has greater oxidative stability than Stellar (see Table XIV).

TABLE XV
Overall Acceptance Scores
and Total Off-Flavor Intensities

<u>Day</u>	<u>Overall Acceptance¹</u>		<u>Total Off-Flavors²</u>	
	<u>IMC 02</u>	<u>Stellar</u>	<u>IMC 02</u>	<u>Stellar</u>
0	8.66	7.75	0.66	0.73
3	6.87	4.18	1.98	7.16
6	7.01	4.57	1.44	6.07
9	6.10	3.67	2.79	8.41
12	4.16	3.67	5.73	7.83

¹Overall acceptance scores are based on a 0-9 scale; 0 is extremely unacceptable and 9 is very acceptable.

²Total off-flavors include fishy, green, cardboard, plastic and painty. Ratings are based on a 0-15 scale, where 0 is bland and 15 is very intense.

(See Figures 8 and 9)

Results: Overall acceptability scores were significantly different after 0, 6 and 9 days ($p=0.05$) (see Table XV).

Correlation between overall acceptance score and total off-flavor intensities is $r^2 -0.9428$ (see Figure 10).

Significantly lower overall acceptance scores and lower off-flavor intensities indicates IMC 02 has significantly better flavor stability than Stellar.

10034698-122701
FOZ227" 8694800T